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SHORT COMMUNICATION

Nucleo-Cytoplasmic Redistribution of the HTLV-I Rex Protein: Alterations by Coexpression of the HTLV-I p21^x Protein

SATOSHI KUBOTA, MASAKAZU HATANAKA,*[,1] and ROGER J. POMERANTZ[2]

The Dorrance H. Hamilton Laboratories, Center for Human Retrovirology, Division of Infectious Diseases, Department of Medicine, Jefferson Medical College, Thomas Jefferson University, 1020 Locust Street, Suite 329 Philadelphia, Pennsylvania 19107; and
**Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan*

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The function of the Rex protein of human T-cell leukemia virus type I (HTLV-I) has been demonstrated to be very similar to the Rev protein of human immunodeficiency virus type 1 (HIV-1). Both of these retroviral regulatory proteins rescue unspliced viral RNAs from the nuclei of infected cells. The Rev protein of HIV-1 has been reported to shuttle between the nucleus/nucleolus and the cytoplasm. Here, we have found that Rex also relocated out of the nucleus in the presence of actinomycin D. This effect was demonstrated in dose- and time-course-dependent manners. In comparison with previous reports on HIV-1 Rev, these effects with Rex seemed to be similar, but less distinct, which may reflect precise differences in the subcellular localization and/or shuttling pathways of Rev and Rex. Interestingly, the endogenous truncated form of the Rex protein, p21^x, significantly interfered with the intracellular translocation of Rex, when coexpressed *in trans.* As expression of p21^x occurs in various HTLV-I-infected cells, p21^x may play a role in the life-cycle of HTLV-I, through regulating the dynamic subcellular distribution of the viral trans-activator, Rex.

The Rex protein of human T-cell leukemia virus type I (HTLV-I) has been regarded as a functional equivalent to the Rev protein of human immunodeficiency virus type 1 (HIV-1). These proteins have been shown to be localized predominantly within nuclei, particularly cell nucleoli (*15, 25*), possess their own nucleolar targeting signals (NOS) (*12, 25*), and are responsible for the cytoplasmic expression of viral structural and enzymatic proteins. Production of these viral proteins are enabled by the cytoplasmic accumulation of their unspliced or partially spliced mRNAs, mediated by the direct interaction of Rex or Rev and the secondary-structured target on their mRNA called the Rex-responsive element (RxRE), or Rev-responsive element (RRE), respectively (*9, 14, 24, 28*).

Further investigations have been performed primarily for the Rev protein. The major function of Rev was shown to be the export of RRE-containing RNA (*7*). Subsequently, several groups have reported, independently, that Rev shuttles between the cytoplasm and nucleus (*5, 6, 11, 17, 23, 27*). However, few reports regarding intracellular shuttling have been presented on Rex (*26*). Using two antisera, which recognize the N-terminus and C-terminus of the Rex protein, we analyzed the distribution and redistribution of Rex in the presence of actinomycin D, which is known to interfere with nuclear translocation of proteins by inhibiting RNA polymerase activity (*22*). We also evaluated the effect of the HTLV-I p21^x protein on the subcellular distribution of the Rex protein.

The p21^x protein can be defined as a natural truncated form of Rex. The initiation methionine of p21^x corresponds to the 79th residue of Rex (Fig. 1). Although the function(s) of p21^x remains unknown, the expression of p21^x was confirmed and p21^x-specific mRNAs were identified in a variety of HTLV-I-infected cell-lines and in primary uncultured cells from adult T-cell leukemia (ATL) patients (*2, 8, 21*). Here, based on the results obtained in this study, a possible role for p21^x in the life-cycle of HTLV-I is suggested.

Two eukaryotic expression plasmids have been constructed to obtain independent expression of Rex and p21 (Fig. 1). Since the p21^x gene is a part of the *rex* gene, we had to introduce a point mutation at the initiation codon of the p21^x gene to avoid concomitant expression of p21^x along with the Rex protein. The Rex expression plasmid, pH2RexdL, produces a Rex protein with an isoleucine residue at residue number 79, instead of methionine, which had been proven to be fully functional (*12*). Construction of these plasmids was previously described (*12, 19*). They possess the same backbone, with an SV40 promoter and origin of replication (Fig. 1). The experiments were performed by transient gene expression and

[1] Present address: Shionogi Institute for Medical Science, Settsushi, Osaka, 566, Japan.

[2] To whom correspondence and reprint requests should be addressed. Fax: (215) 923-1956.

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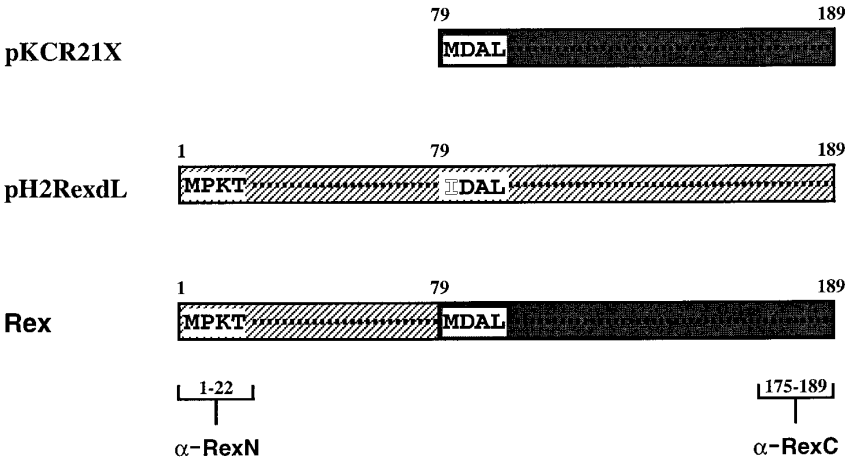

FIG. 1. Primary structures of the Rex-constructs. pKCR21X and pH2RexdL represent eukaryotic expression vectors which express the respective proteins illustrated. The structure of wild-type Rex is shown for comparison. Four amino acid sequences from initiation methionines of Rex and $p21^x$ are displayed as single letter codes. The alternative isoleucine residue in pH2RexdL, which had been introduced by site-directed mutagenesis, is shown in an outlined letter. Numbers denote residues counted from the initiation methionine of the Rex protein. Amino acid sequence-numbers of the synthetic peptides, used for raising each antibody against Rex, are also indicated at the bottom of the figure.

subsequent immunofluorescence analyses. Cos-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Twenty hours prior to DNA transfection, cells were seeded into 8-well Lab-Tek Chamber slides (Nunc, Inc.) at a cell density of 5×10^4/well. For each well, 250 ng of pH2RexdL with or without 250 ng of pKCR21X was transfected by an established DEAE-dextran method with slight modifications (*4*). Forty-eight hours after transfection, cells were treated with 10 μg/ml of cycloheximide and various concentrations of actinomycin D or 100 μM of 5,6-dichlororibosyl benzimidazole (DRB) for 1 to 3.5 hr at 37°. Afterwards, cells were washed with phosphate-buffered saline (PBS), fixed with 3.5% formaldehyde/PBS for 20 min, and permeablized with 0.1% NP-40/PBS for 10 min at room temperature. Two affinity-purified immunoglobulin G (IgG) fractions of rabbit anti-Rex sera were used for detection of intracellular Rex or $p21^x$. The antibodies, which were designated as anti-RexN and anti-RexC, had been raised against synthetic peptides corresponding to residues 1–22, and residues 175–189 of the Rex protein, respectively. Therefore, anti-RexN reacts only with Rex, whereas anti-RexC reacts with both Rex and $p21^x$, as confirmed by radioimmunoprecipitation analysis in a previous report (*1*).

In initial experiments, we analyzed the subcellular distribution of the Rex protein in the absence or presence of actinomycin D at a relatively high dose, while translation was arrested by cycloheximide. As shown in Figs. 2 and 3, treatment with 10 μg of actinomycin D for 2 hr dramatically altered the subcellular distribution of Rex. The nuclear-dominant population decreased and cytoplasmic-dominant cellular population increased with actinomycin D. Thus, Rex is found to migrate out from the cell nucleus. We repeated the same experiment using both antibodies and obtained very similar results (Fig. 3), suggesting the significance and specificity of these results. Of note, the effects observed were somewhat less dramatic than for similar experiments with HIV-1 Rev (*23*).

Then, we performed time-course studies at a fixed concentration of actinomycin D, and also elucidated the dose-dependency of these effects. The demonstration of the nuclear-export of Rex, which was manifested by actinomycin D at a concentration of 10 μg/ml, gradually rose as the incubation time increased until 3.5 hr. However, Rex did not seem to be completely exported out of the nuclei even after 3.5 hr (Fig. 4A). In a series of experiments performed with a fixed incubation time of 2 hr, as the concentration of actinomycin D was decreased, the nuclear-export of Rex was also remarkably attenuated in a dose-dependent manner (Fig. 4B). In fact, at a concentration of 0.1 μg/ml, actinomycin D demonstrated only slight effects on the intracellular redistribution of Rex. These findings are similar, but somewhat different from published data on the HIV-1 Rev protein, in which Rev was expelled out of the nucleus, in most cells, after incubation with 0.04 μg/ml of actinomycin D (*23*). The effect of 100 μM of DRB, which had been shown to specifically inhibit RNA polymerase II activity (*22*), was also evaluated and was comparable to 0.1 μg/ml actinomycin D (data not illustrated). At the present stage, it is not possible to clearly dissect the relationship between intracellular translocation of Rex and specific RNA polymerase activities. High doses of actinomycin D, such as 10 μg/ml, and DRB may cause nucleolar disintegration (*5, 6*), and may affect other biological functions of the cell. Further investigation is required to determine which cellular factor(s) is responsible for the nuclear export of the Rex protein.

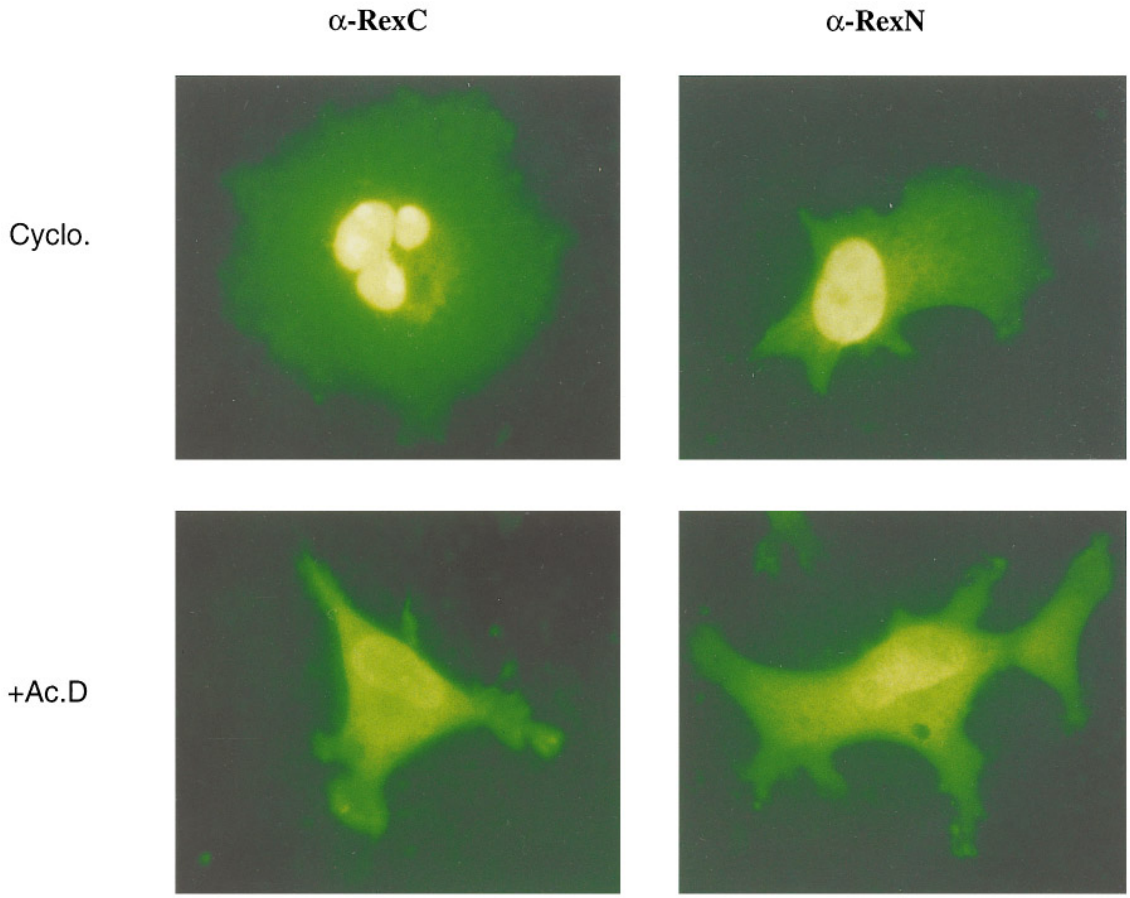

FIG. 2. Representative photomicrographs of the intracellular distribution of Rex with or without actinomycin D treatment. Cos-1 cells were transfected with pH2RexdL, incubated in culture media containing 10 μg/ml of cycloheximide (Cyclo) with or without 10 μg/ml of actinomycin D (Ac. D) for 2 hr at 37°, and stained with either anti-RexN or anti-RexC antibody. (Top) After treatment with cycloheximide only. (Bottom) Treatment with actinomycin D and cycloheximide. (Right) Stained with anti-RexN. (Left) Stained with anti-RexC. Immunofluorescence was obtained by secondary staining with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit polyclonal IgG.

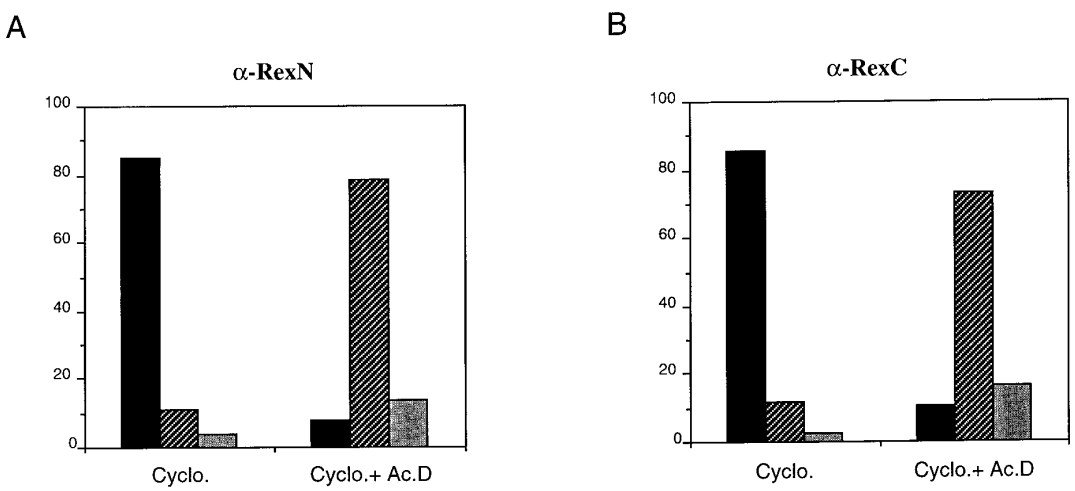

FIG. 3. The intracellular redistribution effects of actinomycin D on the Rex protein. Results from independent experiments, using two different antibodies, are displayed. Cos-1 cells were transfected with pH2RexdL and the distribution pattern of Rex was analyzed by indirect immunofluorescence (IF). More than 50 IF-positive cells were classified into three groups, and each cellular population is represented by the following bars, as percentages of total cells counted. Solid black bar, nuclear $>$ cytoplasmic. Hatched bar, nuclear $=$ cytoplasmic. Stippled bar, nuclear $<$ cytoplasmic. Values after 2 hr incubation with and without 10 μg/ml of actinomycin D, in the presence of cycloheximide, are compared in each panel. (A) Analyses by anti-RexN antibody. (B) Analyses by anti-RexC antibody.

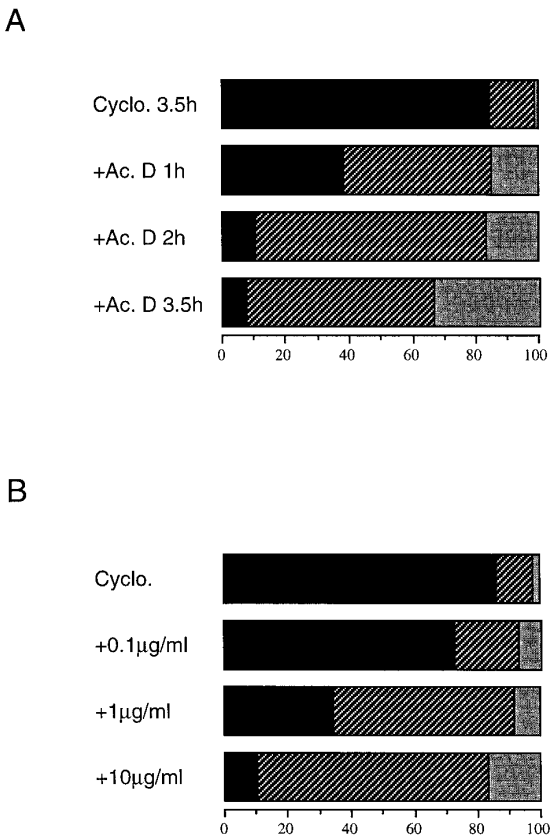

FIG. 4. (A) Time-course studies of the effects of 10 μg/ml of actinomycin D. Cos-1 cells transfected with pH2RexdL were analyzed after variable hours of incubation. (B) Dose-dependency of the effects of actinomycin D on the intracellular distribution of Rex. Cells were treated with each concentration of actinomycin D for 2 hr at 37° and analyzed by indirect immunofluorescence microscopy. More than 50 IF-positive cells, stained with anti-RexC antibody, were classified in three groups as described in the legend of Fig. 1. Each population is represented as percentages of total IF-positive cells. Solid black box, nuclear $>$ cytoplasmic. Hatched box, nuclear $=$ cytoplasmic. Stippled box, nuclear $<$ cytoplasmic. These data are representative of at least two independent experiments for each variable.

The function of $p21^x$ remains unknown; however, a possible antagonistic functional role of $p21^x$ against Rex had been reported (*8*). Therefore, we suspected that $p21^x$ may alter the subcellular distribution of Rex. To address this hypothesis, we coexpressed $p21^x$ with Rex in Cos-1 cells, by transient cotransfection. Since anti-RexN reacts only with Rex, we could analyze the subcellular distribution of Rex in the presence of coexpressed $p21^x$ using this antibody. Expression of $p21^x$, from this plasmid in Cos-1 cells, was previously confirmed by immunofluorescence (IF) and radioimmunoprecipitation analyses (*18*). As shown in Fig. 5, the $p21^x$ protein significantly enhanced the nuclear-localization of the Rex protein, regardless of actinomycin D treatment. The significance of the effect was further demonstrated by extensive analyses. Namely, in complementary experiments, a transfection methodology which is different from the initial experiments was employed (see Fig. 6, legend), and total DNA quantities of each transfection experiment was adjusted equally by the parental plasmid, so as to standardize the expression level of the Rex protein. Examination of the effect of three different doses of the $p21^{x-}$ expression construct has clearly uncovered a dose-dependent, significant effect of $p21^x$ on the subcellular redistribution of the Rex protein, in the presence of actinomycin D (Fig. 6).

We have analyzed the subcellular distribution of the Rex protein and found that it was translocated from the nucleus to the cytoplasm after treatment with actinomycin D. Relatively less strong nuclear export was observed with Rex, at similar doses of actinomycin D, as compared to the Rev protein of HIV-1. This may be due to the differences in the intranuclear localization and shuttling pathways between these two retroviral proteins. Detailed analyses by confocal laser scanning microscopy had shown that Rex is present as nucleolar speckles in an HTLV-I-infected cell-line and can be seen diffusely in the nucleoplasm when highly expressed by transient DNA transfection in Cos-7 cells (*20*). In contrast, Rev accumulates strongly into the nucleolus with very little distribution in the nucleoplasm, even when it was overexpressed (*15*). No Rev-speckle formation was observed and Rev was distributed diffusely within the nucleoli (*20*). These differences could be caused by different modes of interactions between nuclear/nucleolar factors and Rev or Rex.

The Rex protein possesses two distinct domains which are thought to be responsible for nucleo-cytoplasmic shuttling. One is the NOS (residue 1–19), which is required for the nuclear/nucleolar accumulation of Rex, and the other is the so-called leucine-motif/activation domain (residue 82–93) (*16*). This domain is thought to be a nuclear export signal (NES), although there has been no evidence which has clearly demonstrated that the Rex leucine-motif/activation domain is a NES. Of note, the peptide motif of the Rex leucine-motif/activation domain is functionally interchangeable with that of the Rev NES (*10*) and cellular factors that bind to the Rev NES were shown to bind to the leucine-motif/activation domain of Rex as well (*3, 13*). Interestingly, the $p21^x$ protein retains the putative NES at its amino terminus. However, since $p21^x$ is lacking the NOS, it retains no function as Rex. In this study, enhancement of the nuclear localization of Rex by the $p21^x$ protein was observed. A mechanism(s) to explain these effects can be proposed. Owing to the putative NES, $p21^x$ would be able to bind to the cellular cofactors which are responsible for nuclear export. Such binding of these factors to $p21^x$ would not assist in the export of viral RNAs. Consequently, in the presence of $p21^x$, the cellular factors which are responsible for the nuclear export would be consumed. In such circumstances, the efficiency of the nuclear export of Rex would decline and, as a result, more Rex would stay in the

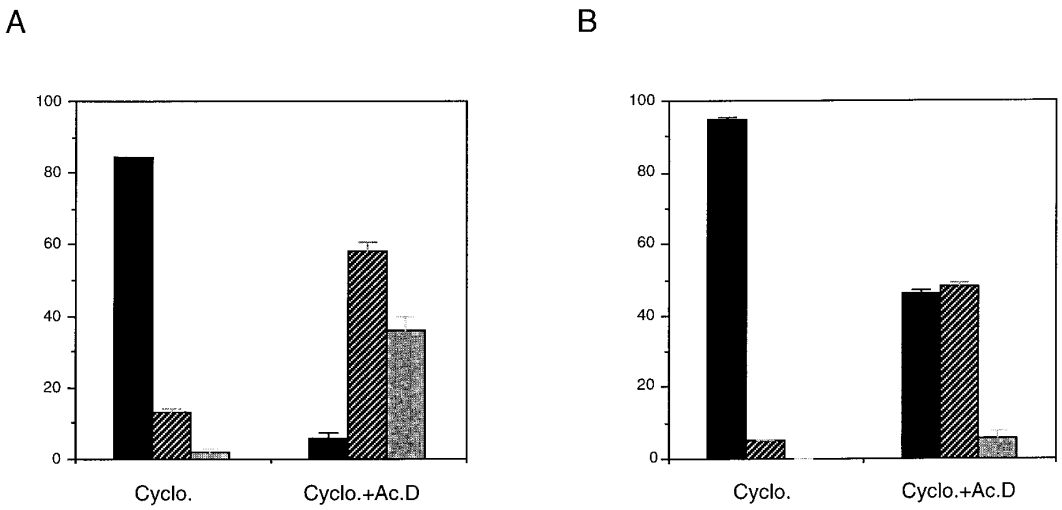

FIG. 5. Effect of the p21^x protein on the subcellular localization of the Rex protein. Transfected Cos-1 cells were treated with 10 μg/ml of cycloheximide alone or with 10 μg/ml of actinomycin D for 3.5 hr at 37°. IF-positive cells, detected by anti-RexN antibody, were analyzed similarly to other experiments (see Fig. 3 legend). Independent experiments were repeated twice and the mean values are shown with error bars. (A) Without coexpressed p21^x. (B) With coexpressed p21^x.

nucleus. Thus, the observed enhancing effect of the nuclear accumulation of Rex may be due to the titration of cellular factors by p21^x, via their common leucine-motif/activation domain.

The expression of p21^x has been confirmed not only in HTLV-I-infected cell-lines (*8, 21*), but also, importantly, in primary uncultured ATL cells (*2*). Alternatively spliced monocistronic mRNAs only for p21^x expression have been isolated and characterized in those studies. Correlation of these mRNA levels and the level of p21^x expression has also been confirmed by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blotting (*8, 21*). Thus, p21^x does not seem to be a mere unnecessary, deleted form of Rex, which is formed by inappropriate translational initiation on Tax/Rex mRNAs, but may be an independent regulatory protein, supplied *in trans,* from its own mRNAs. In this study, we supplied p21^x *in trans,* and found a repressive effect against Rex-shuttling. Taken together with a weak inhibitory effect of p21^x against Rex function, which was previously described (*8*), these findings imply some functional roles for p21^x in the life-cycle of HTLV-I. It can be hypothesized that p21^x may be involved in a less prominent, but more delicate regulation of HTLV-I gene expression, as compared to certain other viral regulatory proteins. As such, it may be involved in the maintenance of the long latency period of HTLV-I-infected-individuals.

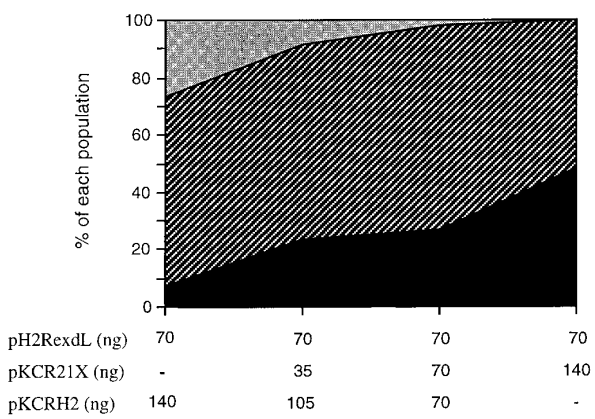

FIG. 6. Dose-dependent alteration of the relocation of Rex by p21^x after actinomycin D treatment. Cos-1 cells were transfected with each mixture of DNA (total-210 ng), which is shown on the horizontal axis, by a liposome-mediated DNA transfer system (Lipofectamine; GIBCO/BRL), according to the manufacturer's optimized protocol. The plasmid entitled pKCRH2 is the parental plasmid, without exogenous cDNA. Forty-eight hours posttransfection, cells were treated with 10 μg/ml of cycloheximide and actinomycin D for 3.5 hr at 37°. Immunofluorescence analyses and quantification were carried out, via the same procedure as described in the legend of Fig. 5. Solid black area, nuclear $>$ cytoplasmic. Hatched area, nuclear $=$ cytoplasmic. Stippled area, nuclear $<$ cytoplasmic. Data shown here are the mean values of two independent sets of experiments.

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